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MEMO associated with an ErbB2 receptor phosphopeptide reveals a new phosphotyrosine motif

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ABSTRACT

Tyrosine phosphorylations are essential in signal transduction. Recently, a new type of phosphotyrosine binding protein, MEMO (Mediator of ErbB2-driven cell motility), has been reported to bind specifically to an ErbB2-derived phosphorylated peptide encompassing Tyr-1227 (PYD). Structural and functional analyses of variants of this peptide revealed the minimum sequence required for MEMO recognition. Using a docking approach we have generated a structural model for MEMO/PYD complex and compare this new phosphotyrosine motif to SH2 and PTB phosphotyrosine motives.

Structured summary of protein interactions:
ErbB2 physically interacts with **MEMO** by pull down (View interaction 1, 2, 3, 4, 5, 6)

1. Introduction

Receptor tyrosine kinases are major players in transduction of extracellular cues into intracellular signals that allow a cell to adjust to the environment. Members of the ErbB family are transmembrane receptor kinases expressed in many different tissues. These epidermal growth factor receptors play a crucial role in cell growth, differentiation and migration. Aberrant expression of receptor tyrosine kinases leads to dramatic physiological consequences such as malignant tumor formation. For instance, overexpression of the ErbB2/Her2/Neu receptor tyrosine kinase in breast cancers is associated with the most aggressive tumors [1,2]. Experimental studies have revealed the contribution of ErbB2 to tumor formation and ErbB2 is now considered as a therapeutic target in breast cancer [3]. Ligand binding to the extracellular regions of the ErbB receptors induces the formation of specific homo- or heterodimeric receptor complexes, leading to kinase activation followed by transphosphorylation of specific tyrosine residues in the cytoplasmic tail.

Src homology 2 (SH2) and phosphotyrosine binding (PTB) domains are the major domains recognizing phosphotyrosine (pTyr) residues in receptor tyrosine kinases [4,5]. The overall structure of PTB domains and the mechanisms by which they bind both pTyr residues and associated proximal amino acid residues differ dramatically from SH2 domains. While SH2 domain binding is strongly dependent on the presence of both the pTyr residue and adjacent amino-acids carboxy-terminal to the pTyr residue, recognition residues for PTB domains are located amino-terminal to the pTyr [6,7]. Systematic search for proteins associating with phosphorylated tyrosine residues of ErbB cytoplasmic domains indicated that most ErbB2-binding proteins harbor one of the two types of domains [8]. However, recently, MEMO (Mediator of ErbB2-driven cell motility) was described as a new class of phosphotyrosine binding protein that binds to a phosphorylated tyrosine (Tyr1227 in rat, Tyr1222 in human, also referenced as TyrD) encompassing peptide of ErbB2 [9]. MEMO is an effector of ErbB2 involved in breast carcinoma cell migration [10]. MEMO contributes to localize the small G protein RhoA and its effector mDia1 at the plasma membrane and controls microtubules dynamics, formation of adhesion sites and recruitment of

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actin-binding proteins [11]. Pull down experiments using a recombinant protein demonstrated that MEMO can directly bind to pTyrD-containing peptide (PYD1–16), in the absence of additional factors [9]. Analysis of the primary sequence or the 3D structure of MEMO does not reveal the presence of PTB or SH2 domains. However, MEMO is structurally related to bacterial dioxygenases [9]. Although, it was postulated that MEMO binds to the phosphorylated TyrD through its vestigial active site, its mode of interaction with the phosphorylated peptide could not be described by X-ray. We have generated a model of the complex using a restrained-docking approach [12–14].

2. Materials and methods

2.1. Peptide synthesis

Seven peptides derived from PYD1–16 were produced by EZBiolab: PYD1–16 (SPAFDNLYpYWDQDPPE) and YD1–16 (SPAFDNLYYWDQDPPE) peptides correspond to amino-acids 1214–1229 from human ErbB2 phosphorylated or not on Tyr1222; PYD1–10 (SPAFDNLYpYW), PYD4–13 (FDNLYpYWDQD) and PYD7–16 (YpYWDQDPPE) are truncated peptides; $PYD_{D/A}$ (SPAFANLYpYWAQAPPE) is a triple mutant of PYD1–16 (D5A,

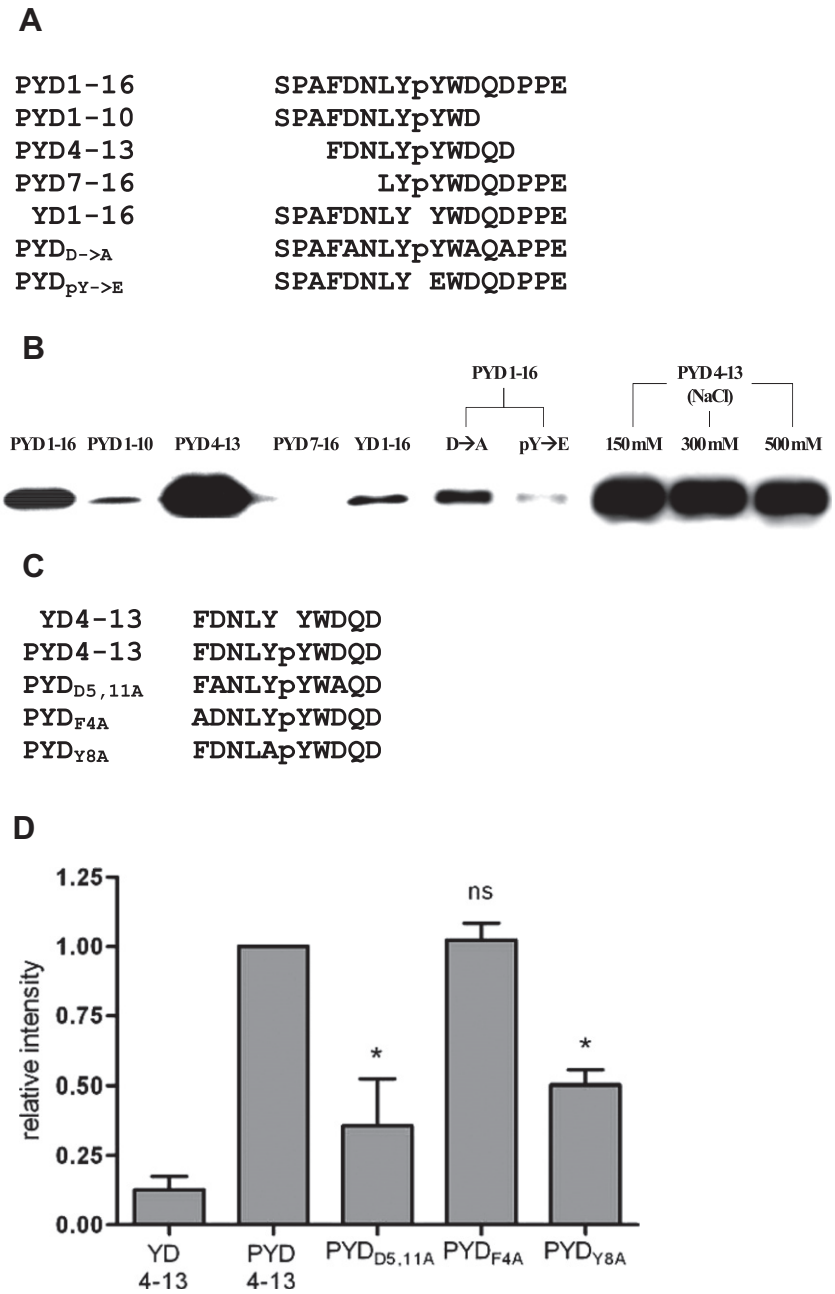

Fig. 1. (A and C) Sequences of PYD1–16, and PYD4–13, truncated and mutant peptides used in (B and D). (B) Peptide binding activity of MEMO to PYD1–16 variants. Pulldown experiments showing the binding of MEMO to the TyrD containing peptides shown in (A). Biotinylated peptides, incubated with MEMO-expressing SKBr3 cell lysates, were pulled down using streptavidin-sepharose and analyzed by Western blotting using anti-MEMO antibodies. (D) The amount of MEMO bound to the peptides indicated in (C) was quantified by densitometric analysis of the Western blots. Results were expressed relative to control PYD4–13 peptide. Mean and S.E.M. of three independent analyses are represented. $^{*}P < 0.05$, one way ANOVA-Bonferroni analysis.

D11A and D13A); finally, $PYD_{pY/E}$ (SPAFDNLYEWDQDPPE) is a mutant of PYD1–16 in which the pTyrD (pTyr9) residue was replaced by a glutamate residue. Three peptides derived from PYD4–13 were synthesized by Schafer-N: PYD_{F4A} (ADNLYpYWDQD) has the F4A mutation, PYD_{Y8A} (FDNLApYWDQD) has the Y8A mutation and $PYD_{D5,11A}$ (FANLYpYWAQD) has both D5A and D11A substitutions. All the peptides carried a biotin group at the N-terminal extremity.

2.2. Pulldown experiments

Phosphorylated and non-phosphorylated peptides were coupled via the N-terminal biotin group to streptavidin sepharose (GE Healthcare) for 1 h in 10 mM sodium phosphate buffer pH 6.0. The beads were then mixed with lysates of MEMO-expressing SKBr3 cells, in the same buffer for 2 h at room temperature. Proteins bound to the beads were subjected to SDS–PAGE and Western blotting with MEMO antibodies, as previously reported [9]. To observe the effect of ionic strength on MEMO/PYD1–16 interaction, complex formation was performed in the presence of 150 mM, 300 mM or 500 mM NaCl.

2.3. NMR measurements

Proton assignment was obtained using COSY, TOCSY and NOESY experiments recorded at 300 K on a Bruker Avance 600 spectrometer using a 2 mM peptide sample in 10 mM sodium phosphate buffer pH 4.5 including 10% D_2O. For structure calculation homonuclear 2D-NOESY spectra were recorded using various mixing times (80, 150 and 250 ms). The program TOPSPIN 2.1 was used for data collection and processing. CARA software was used for spectral analysis [15]. Proton assignment of PYD1–16 was deposited at the BMRB (BMRB16495).

2.4. Structure calculation

On the basis of sequential and medium range nOes, the structure calculation of PYD1–16 was performed with the program CYANA 2.1 using a tyrosine residue instead of the phosphotyrosine [16]. A family of 15 structures was retained. The phosphorylation of pTyr9 (pTyrD) was generated using the lowest energy conformation and CHARMMforce-field [17] on the CHARMM-GUI web-based graphical interface (http://www.charmm-gui.org). Electrostatic potential was obtained using PBEQ solver [18]. Structures were represented using PyMOL program (http://www.pymol.org).

2.5. Docking experiments

HADDOCK (High Ambiguity Driven protein–protein DOCKing) version 2.0, uses biochemical or biophysical data as ambiguous interaction restraints [14]. The software has been implemented in CNS for structure calculations. The docking protocol requires MEMO (PDB ID 3BCZ) and PYD1–16 (PDB file of the lowest energy conformation) PDB files and ambiguous interaction restraints. The experimental restraints were obtained from the previous mutants for MEMO (Trp16, His49, His81, His192 and Cys244) and pTyr9 for the phosphopeptide. The docking consists of three stages: randomization of orientations and rigid body energy minimization, semi-rigid simulated annealing in torsion angle space and final refinement in Cartesian space with explicit solvent. During simulated annealing and water refinement, the amino acids are allowed to move to optimize the interface packing. The final structures are clustered using pairwise backbone RMSD at the interface and analyzed according to their average interaction energies and their average buried surface area.

3. Results

3.1. Identification of the interacting sequence

We have generated a series of PYD1–16 derived peptides and used pulldown experiments to investigate the binding of these peptides to MEMO. Replacement of pTyr9 by either unphosphorylated tyrosine or glutamate induced a drastic reduction of the complex formation, confirming that the phosphotyrosine is essential for MEMO/PYD1–16 interaction (Fig. 1A and B). Then, we have generated three truncated peptides (PYD1–10, PYD4–13 and PYD7–16). Pull down experiments with PYD1–10 showed that deletion of the last six residues at the C-terminus led to a drastic decrease in MEMO binding (Fig. 1A and B). Interaction with

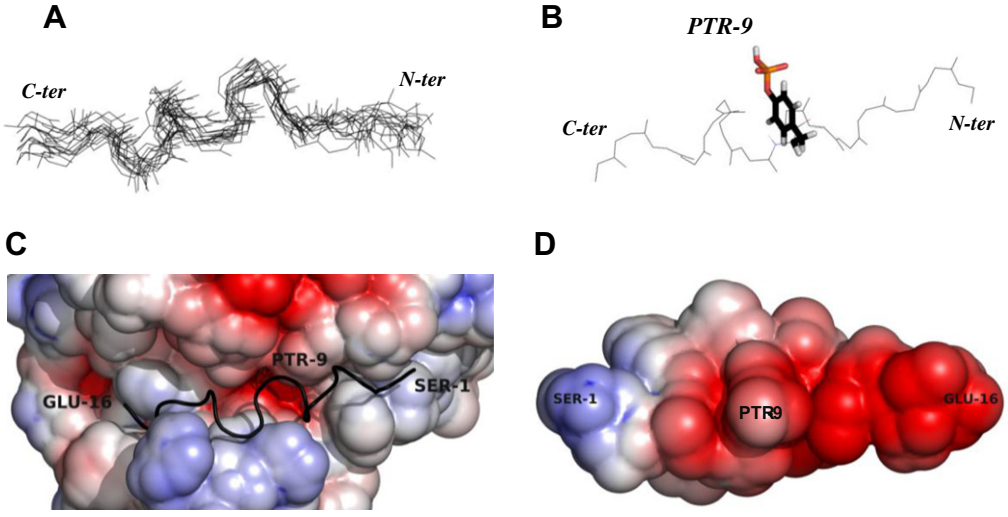

Fig. 2. Structure of pTyrD-containing ErbB2 peptide. (A) Ensemble of the backbone traces of the 15 lowest energy conformers of PYD1–16. (B) Backbone trace of the lowest energy conformer of PYD1–16. Phosphotyrosine side chain of pTyr9 is shown in stick representation and annotated PTR-9. (C) Electrostatic potential surface interaction of MEMO. PYD1–16 peptide is shown in backbone trace. (D) Electrostatic potential surface of PYD1–16. Acidic residues (Asp5, Asp11, Asp13, Glu16 and pTyr9 (PTR-9)) are shown in red and basic residues in blue.

PYD7–16 which contained the phosphotyrosine and the C-terminal residues was also drastically dropped (Fig. 1A and B) demonstrating that the residues located at the N-terminus of the peptide were essential for peptide recognition by MEMO. However, pull down experiments with the PYD4–13 peptide revealed an affinity for MEMO similar to the PYD1–16 phosphopeptide indicating that this core sequence is required for MEMO/PYD interaction.

3.2. Structure determination of the pTyrD-encompassing ErbB2 peptide

The structure of PYD1–16 peptide was investigated by NMR. Assignment of 15 spin systems was obtained from COSY, TOCSY and NOESY spectra. On the basis of 62 sequential and 19 medium range nOes (Figs. S1 and S2), the structure of PYD1–16 was calculated with CYANA. A family of 15 structures has been retained with an average pairwise rms deviation of 1.77 Å (Table S1 and Fig. 2). The average structure indicated that the peptide contained a helical structure from residues Phe4 to Trp10 (numbering corresponds to the positions in the PYD1–16 peptide). Phosphorylation of pTyr9 was obtained using CHARMM-GUI web interface [19].

3.3. Structural model of MEMO/PYD1–16 complex

On the basis of MEMO X-ray structure (3BCZ) and PYD1–16 NMR structure, we have performed docking experiments using HADDOCK [14]. In the 200 models, the phosphotyrosine is located in the large cleft of MEMO, indicating that this interaction is essential for the complex formation. We retained the most favorable solution in terms of intermolecular interaction energy (Fig. 3).

Table 1
Intermolecular hydrogen bonds found in MEMO/PYD complex.

	MEMO		PYD		Distance (Å)
Hydrogen bonds	His192	HE2	pTyr9	O1P	1.81
	Cys244	HG	pTyr9	O1P	2.12
	His49	HE2	pTyr9	O3P	1.61
	His81	HD1	pTyr9	O3P	1.57
	Trp16	HE1	Phe4	O	2.35
	Tyr54	OH	Asp5	OD2	1.68
	Arg196	HH11	Asn6	OD1	2.44
	His 82	HE2	Tyr8	OH	1.82
	Arg198	HH12	Asp11	OD2	1.73
	Arg198	HH21	Asp13	O	1.89
	His81	HE2	Asp13	OD1	1.59
	H192	HD1	Asp13	OD2	1.64
	His240	HE2	Glu16	OE1	1.68

In this model, the elongated phosphopeptide is located in a large groove of MEMO structure, resulting in an interacting surface of 926.4 Å^2 (Fig. 3A and B). The charged surface of the peptide including pTyr9 is anchored to MEMO interface through hydrogen bonds (Fig. 3C and D). The interface analysis was done using PISA server [20] and VMD. Intermolecular hydrogen bonds resulting from the complex formation are summarized in Table 1. Fig. 3C highlights the hydrogen bonds involved in the phosphate group stabilization. The three histidine residues (His49, His81 and His192) of the vestigial dioxygenase active site, and also Cys244 form hydrogen bonds with oxygen atoms of the phosphate group. The three histidine residues have the same orientation as they have

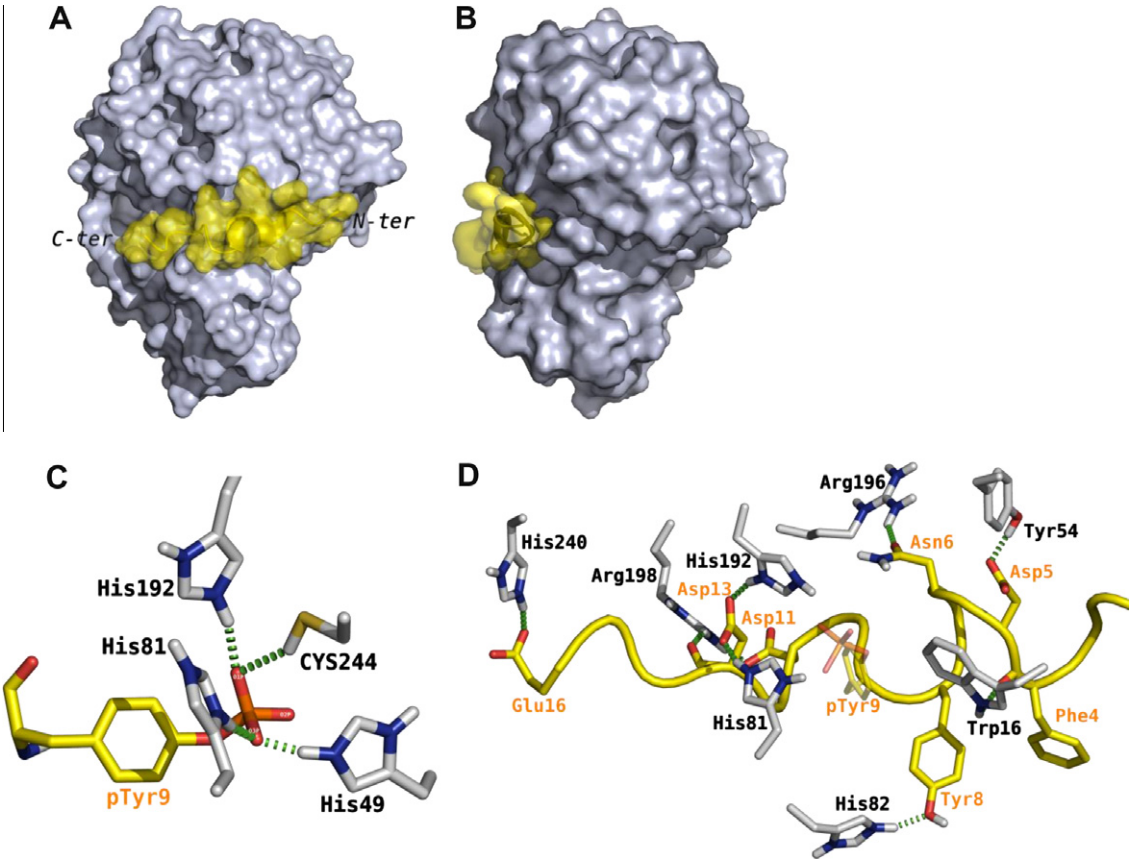

Fig. 3. Best structural model of MEMO/PYD1–16 complex resulting from HADDOCK. (A) Surface representation of the complex; PYD1–16 is shown in yellow. (B) Side view of the complex corresponding to a rotation of 90° compare to (A). (C) Closer view of the interacting site showing hydrogen bonds associated to the phosphate group of pTyr9, in yellow. (D) Closer view of the interacting site indicating side chain intermolecular interactions. PYD1–16 is in yellow.

in Ygid active site, and the phosphate group is found at the same position as the metal in LigB and Ygid. MEMO Trp16 known to play a major role in PYD1–16/MEMO interaction [9] was found in the environment of the phosphate group and no far of MEMO Phe4; however, PYD_{F4A} substitution did not affect the complex formation indicating that MEMO Trp16/PYD Phe4 interaction is not essential for the binding. Fig. 3D shows that residues Ser1 to Ala3 and Pro14 to Glu16 of PYD1–16 are weakly involved in protein–protein interactions; these data are in very good agreement with our results on pull down experiments performed on the short derived peptides that indicated PYD4–13 as a minimum required sequence. Concerning the hydrogen bonds found at the complex interface (Table 1), even if we observed poor effects of salts on the complex formation, double and triple mutations of Asp5, Asp11 and Asp13 revealed significant effects on the complex formation, underlining the implication of these residues at the interaction site (Fig. 1). These data are in agreement with the minimal length found for ErbB2 peptide from Phe4 to Asp13, including Asp5, Asp11 and Asp13. PYD Asp5 is involved in a hydrogen bond with MEMO Tyr54. Previous replacement of this residue was found not essential but diminished pYD binding [9]. PYD Asp13 is found in a strong interaction with His81 and His192, two ligands of the pTyr phosphate group, being essential for the complex formation. Concerning PYD Glu16, the high affinity of PYD4–13 for MEMO suggests a weak importance of this residue in the complex formation. Finally, the length of the hydrogen bond observed between MEMO Arg196 and PYD Asn6, and the observation that MEMO Arg196 mutation did not affect the complex [9], suggest that this bond is not essential for MEMO/ErbB2 interaction. It is to be noticed that PYD_{Y8A} substitution induces an important effect on the complex formation indicating that this residue is very important for the interaction.

4. Discussion

Phosphorylation on tyrosine residues creates binding sites for modular phosphoprotein-binding domains to form multiprotein complexes, and to provide a reversible way to regulate protein–protein interactions both spatially and temporally [6]. ErbB2 receptor is a multidomain protein containing an extracellular domain, a transmembrane helix and an intracellular region constituted of a kinase domain and an additional unstructured C-terminal tail [21]. Although the structures of the extracellular and kinase domains have been determined, no structural information on the extended peptide has been reported. Analysis of the sequence surrounding the five main autophosphorylation sites in ErbB2 C-terminal extremity [22] shows that pTyrB is part of a SH2 consensus sequence (pYVNQ), while pTyrC and pTyrE are included in a PTB consensus sequence (XNPXpY). In contrast, pTyrD is included in a distinct sequence motif (FDNLYpYWDQD), which results in a well structured peptide that allow interaction with MEMO, a new type of phosphotyrosine binding protein [9].

The pH-dependence of MEMO/PYD1–16 binding suggested that histidine residues were particularly important for the interaction and may balance the charge of the phosphorylated tyrosine. A large cleft is present in MEMO structure and amino acid substitutions of histidine residues in this cleft, strongly diminished PYD1–16 binding [9]. Our study describing the structure and minimal sequence requirement for PYD1–16 ErbB2 peptide binding to MEMO provides valuable insights for the understanding of MEMO–PYD1–16 interaction. The present data clearly demonstrate that a phosphotyrosine residue is essential for MEMO/PYD1–16 interaction, and that additional interactions involving PYD Asp5, Tyr8, Asp11 and Asp13 are very important for the complex formation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2011.07.048.

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